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THE PHYLOGENY AND MORPHOLOGICAL ADAPTATIONS OF *CYCLOTUS TAIVANUS* SSP. (GASTROPODA: CYCLOPHORIDAE)

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ABSTRACT

By traditional classification, there are five *Cyclotus taivanus* subspecies in the low mountainous area of Taiwan and Okinawa: *C. taivanus adamsi*, *C. t. dilatus*, *C. t. diminutus*, *C. t. peraffinis*, and *C. t. taivanus*. The molecular phylogenetic relationships of this group have never been discussed. In order to investigate the relationships between *C. taivanus* ssp., we sequenced part of the mitochondrial COI and the 16S rRNA gene from 26 sampling sites. We also measured 9 shell traits for morphological analysis. Even though morphological PCA analysis revealed a more or less continuous distribution of individuals in morph-space, the two highly divergent haplotype clades in molecular analysis indicated the presence of two independently evolving lineages. Our results indicated that the sequence divergence between the two independent clades was almost as high as that among other Cyclophoridae species found previously. Therefore, from the viewpoint of taxonomy, *C. t. adamsi* should be considered a valid species, and we here raise the current taxon to a full species: *C. adamsi*. By environmental analysis, temperature was found to be a limiting factor in the distribution of *C. adamsi* and the *C. taivanus* group (*C. t. dilatus*, *C. t. diminutus*, *C. t. peraffinis*, and *C. t. taivanus*). The ecological divergence is probably a rule of speciation in our case. The PLS (Partial least square) analysis results indicate that phenotypic plasticity may be a key element of the variable shell in the *C. taivanus* group. The speciation process is not complete among the *C. taivanus* group, and the adaptation to climatic pressure continues to be a rule of the speciation process.

Key words: phylogeny, morphological adaptations, *Cyclotus taivanus*, biogeography, Taiwan.

INTRODUCTION

Cyclotus taivanus ssp. is widely distributed in Taiwan and the Japanese island of Okinawa. Traditionally, five subspecies have been described. The north Taiwan subspecies *C. t. adamsi* (Pilsbry & Hirase, 1905) has a tall spire with a uniform yellow or tawny yellow color, although some individuals show a reddish-brown zigzag pattern. The other four subspecies have flat spires, but are characterized by a wide extended lip in the east Taiwanese subspecies *C. t. dilatus* Lee & Wu, 2001, a very narrow lip in the Lanyu Island subspecies *C. t. diminutus* Lee & Wu, 2001, an intermediate lip in the south Taiwanese subspecies *C. t. taivanus* H. Adams, 1870, and a very polished periostracum in the Okinawan subspecies *C. t. peraffinis* (Pilsbry & Hirase, 1905) (which is otherwise similar to *C. t. diminutus*) (Lee & Wu, 2001).

Although, some microsnails can potentially achieve long-distance colonization through passive dispersal (Kirchner et al., 1997; Wada et al., 2011), most land snails have a low vagility (Anderson, 2007; Murray & Clarke, 1984; Thomaz et al., 1996), and therefore cannot avoid unfavorable conditions by migration but are doomed to extinction (Akçakaya & Baur, 1996). Landslides and debris flows often occur in Taiwan due to heavy orogenic movements, the fragile geological environment, earthquakes, typhoons and heavy rainfall (Lin et al., 2004; Yeh, 2006). The snails may isolate in relative stable microhabitat and raise their genetic variance. These render land snails an ideal model for examining the role of speciation history in our place.

The evolutionary processes shaping the phenotypic variation among and within related landsnails have recently become a focus of research interest (Booth et al., 1990; Gould et al., 1974; Gould & Woodruff, 1978; Pfenninger et al., 2003; Pfenninger & Magnin, 2001; Teshima et al., 2003; Woodruff & Gould, 1987). Two ba-

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sic approaches have been taken to the study of variation in shell shape. One approach involves analysis of the distribution of shapes among taxa and hypothesizing about the causes affecting the frequencies of certain shapes (Raup, 1966), while the second approach involves study of the correlations between variation in shape and environmental variation (Goodfriend, 1986). In earlier times, biologists and taxonomists discriminated species based on anatomy and morphology. On the beginning of the 1970s, molecular techniques have been used as an aid for delimiting species (Michael, 1996). Mitochondrial DNA has an important advantage in phylogeny reconstruction and species delimitation (Wiens & Penkrot, 2002). However, malacologists have lagged behind other systematists in applying molecular techniques for delimiting species (Davis, 1994). The last decade, mtDNA data sets have been widely used as markers for delimiting mollusc species (Collin, 2005; Lapègue et al., 2002; Malaquias & Reid, 2008; Meyer, 2003). Further, the phylogeny and evolutionary processes of *C. taivanus* ssp. have not been investigated previously.

The objectives of this study were to use nucleotide sequence information from COI and 16S rRNA gene fragments to investigate the quantitative morphological differences between the subspecies of *C. taivanus*. In particular, we focus on four questions: (1) Does the observed phenotypic variation correspond to different evolutionary lineages? (2) How is the phenotypic variation distributed within and among populations in relation to the variations in COI and 16S? (3) Can we identify environmental variables that co-vary with population differences in morphology? (4) What is the cause of the speciation process?

MATERIALS AND METHODS

Populations Sampled

In total, 26 populations were examined in Taiwan (Fig. 1; Table 1). We did not obtain any living specimens (sampling site 2) or adult individuals (sampling site 5, 18) at some sampling sites. Morphological analysis was performed on 105 adult individuals from 24 locations and DNA analysis on 87 individuals from 25 locations. Both data sets overlapped for 70 individuals.

Morphological Analysis

Individuals were photographed using a Canon D1 digital camera from the aperture view, and eight shell characters were measured using ScopePhoto software (ScopeTek, 2007): H (height), W (width), AW (aperture width), AH (aperture height), BW (body whorl width), BH (body whorl height), LW (outer lip width), UW (umbilicus width) and HN (whorl number) (Fig. 2). H × W, H/W, AW/W, AW/AH, UW/W, LW/AW, BH/BW and (W–UW–LW)/HN were calculated as indices of size, tallness/flatness, aperture proportion, aperture shape, umbilicus proportion, outer lip proportion, flatness degree of body whorl and degree of whorl increase, respectively. These shell morphological variables and sampling site environmental characterization (warm season mean temperature, cold season mean temperature, annual mean temperature, annual range of monthly normal temperature, warm season amount of precipitation, cold season amount of precipitation, annual amount of precipitation, altitude, obtained from Taiwan Central Weather Bureau) (Lin, 1990) were used to perform principal component analysis (PCA) using the PCA option of the XLSTAT package (2010).

PLS analysis was performed using the PLS option of the XLSTAT package (2010) to assess the correlations between quantitative shell traits and environmental variables for each population.

DNA Preparation and Sequencing

The shells and soft parts were separated at the laboratory. Shells were cleaned thoroughly for identification and measurement of shell characters, and soft parts were stored at -80° until DNA extraction. DNA was extracted from the columellar muscle of separate individuals using a slightly modified TEK-based protocol (Jiang et al., 1997). Tissue was placed in TEK buffer (12.5 mM Tris-HCl pH 7.3, 2.5 mM EDTA, 0.4% KCl), then ground with a glass pestle and incubated at 57°C with 20 µl of proteinase K (20 mg/ml) for more than 2 hrs. Tissue was extracted at least twice with phenol and chloroform. 400 µl DNA extract was precipitated by adding 1,000 µl pure ice-cold ethanol and was stored at -20°C for 20 min. DNA was then pelleted by centrifugation for 30 min. After rinsing with 70% ethanol, DNA was resuspended in distilled water and stored at -80°C for DNA amplification.

TABLE 1. List of *Cyclotus* sampling sites used in this study.

	Location	Position	Morphological analysis	DNA ananlysis
1	Guan-in Mountain, Taipei County	25.135861N, 121.428861E	1	1
2	Chih-shan-yin, Taipei City	25.104833N, 121.529889E	3	–
3	The Cao-ling Historic Trail, Iran County	24.993194N, 121.925639E	1	6
4	Kai-cheng Temple, Iran County	24.813139N, 121.710583E	5	7
5	Sheau-jiau-shi, Iran County	24.805833N, 121.704417E	–	2
6	Fwu-shan, Taipei County	24.795750N, 121.492944E	3	1
7	Ren-tzer, Iran County	24.545611N, 121.507278E	1	1
8	Dung-aw-li, Su-aw, Iran County	24.521194N, 121.824889E	5	4
9	Iriomote Island	24.349000N, 123.805000E	5	3
10	Chorng-der, Hualian County	24.189278N, 121.660889E	10	9
11	Chin-heng Bridge, Taroko valley, Hualian County	24.171167N, 121.560417E	2	1
12	Charn-guang Temple, Taroko valley, Hualian County	24.159250N, 121.606111E	2	1
13	County Highway No. 136, 39.5K, Taichung County	24.099361N, 120.790389E	1	1
14	County Highway No.129, 20.5k, Jia-yi County	23.342500N, 120.691861E	7	8
15	Tu-di-gong Temple, Guan-tzy-liing, Tainan County	23.339306N, 120.505556E	5	7
16	Provincial Highway No.20, 49k, Nan-huah, Tainan City	23.100389N, 120.481556E	1	1
17	Shuang-liou Wood Park, Ping-dung County	22.217889N, 120.803000E	1	2
18	Jang-hu, Jia-yi County	23.599333N, 120.656472E	–	1
19	Provincial Highway No.149, Yun-lin County	23.589639N, 120.569306E	2	1
20	Bau-Lai, Kaohsiung County	23.108639N, 120.699722E	21	3
21	Shan-ping, Kaohsiung County	22.965917N, 120.683944E	7	9
22	Liou-guei, Kaohsiung County	22.925222N, 120.652833E	6	1
23	Hae-shern Temple, San-dih-men, Ping-dung County	22.819972N, 120.640444E	2	3
24	Wu-tai, Ping-dung County	22.751528N, 120.727167E	1	1
25	Wu-tai, Ping-dung County	22.743167N, 120.706944E	3	3
26	Yongsing, Lanyu Island, Taidung County	22.028222N, 121.579667E	10	10

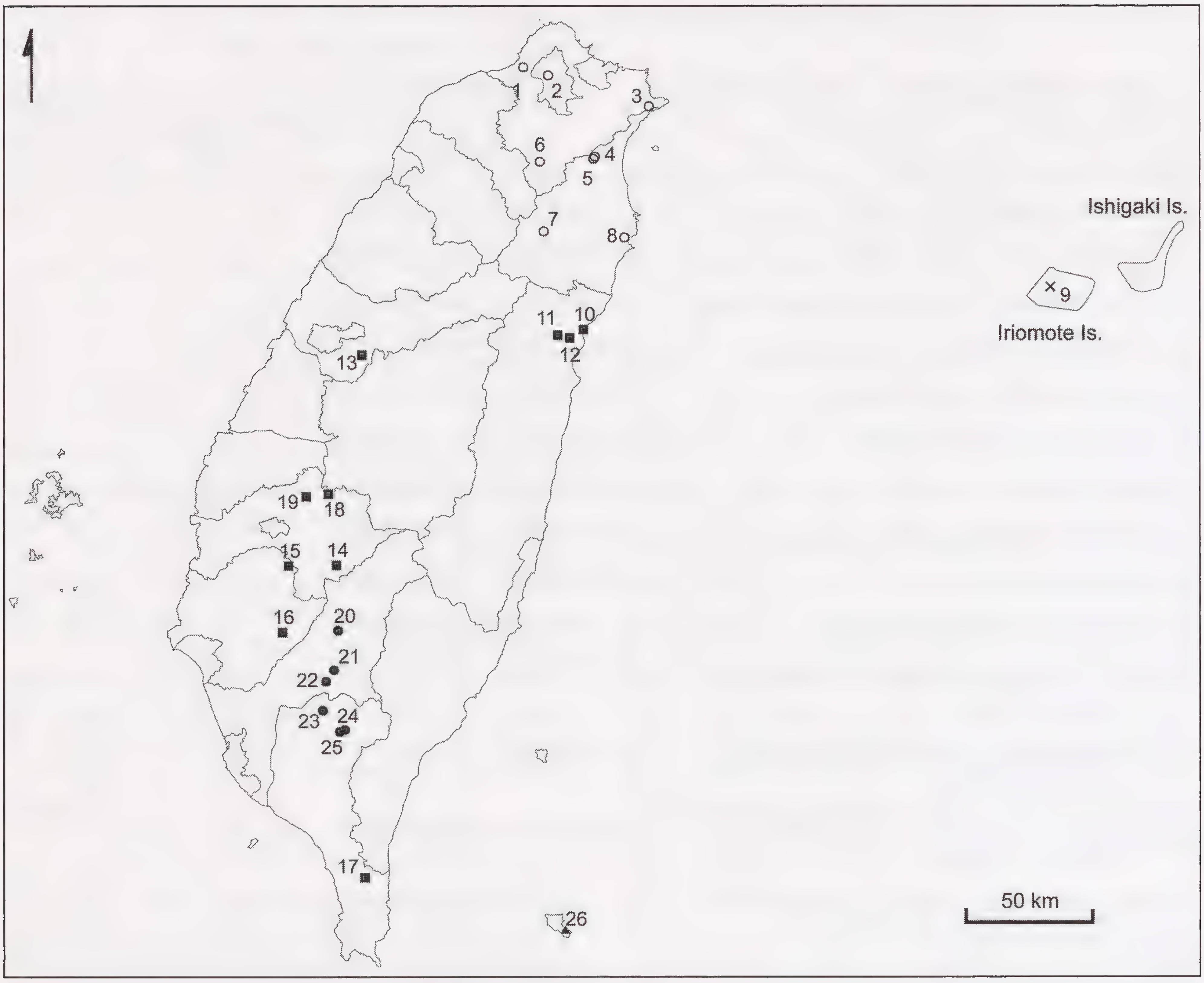

FIG. 1. Geographic range of the *Cyclotus* species complex. Different symbols illustrate the sampling sites of the five taxa. Open circle: *C. taivanus adamsi*; solid square: *C. t. dilatus*; solid circle: *C. t. taivanus*; solid triangle: *C. t. diminutus*; X: *C. t. peraffinis*.

An approximately 860-bp fragment of the mitochondrial 16S rRNA gene was amplified by PCR using primers 16SRT (5'–ACA TAT CGC CCG TCA CTC TC–3') and 16SL900 (5'–AAA TGA TTA TGC TAC CTT TGC–3'), and an exactly 531-bp fragment of the COI gene was amplified using primers LCO1490 (5'–GGT CAA CAA ATC ATA AAG ATA TTG G–3') and HCO2198 (5'–TAA ACT TCA GGG TGA CCA AAA AAT CA–3') (Folmer et al., 1994). For COI some individuals got poor PCR products using primers LCO1490 and HCO2198. For those we use primer CoL90 (5'-TAG TGT TAA AAT TAC GAT CAG T-3') and CoR600 (5'-AAG TCT TCT AAT TCG TGC AGA A-3'). The PCR reactions contained 10–50 ng/µl template DNA, 10 pmol of each primer, 5 µl 10× reaction buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% gelatin, 1% Triton X-100), 0.4 µl 25 mM/µl dNTP, 0.2 µl 50 mM Mg^{2+}, and 0.4 µl Taq polymerase (5 unit/µl) in a total volume of 50 µl. Thermal cycling for 16S rRNA was performed with an initial denaturation for 5 min at 95°C, followed by 30 cycles of 30 s at 95°C, 45 s at 57°C, 50 s at 72°C and final extension at 72°C for 10 min, final holding at 4°C. Thermal cycling for COI was performed similarly, with the exception that the annealing temperature was changed to 47°C. The PCR products were then purified using a purification kit (AMP PCR purification, Beckman) and then sequenced using an ABI 3700 autosequencer.

Phylogenetic Analyses

The COI + 16S rRNA data of *Cyclotus taivanus* ssp. were combined with data of the outgroup *Ptychopoma wilsoni* (Pfeiffer, 1865), *Cyclophorus moellendorffi* Schmacker & Boettger, 1891,

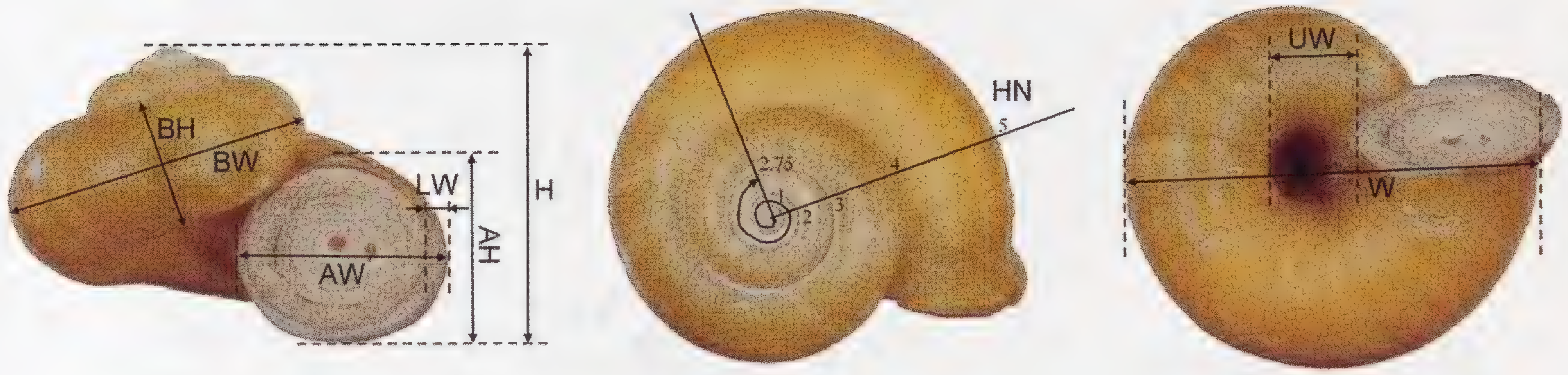

FIG. 2. The morphological characters of *Cyclotus*. AH = aperture height, AW = aperture width, BH = body whorl, BW = body whorl width, H = height, HN = whorl number, LW = outer lip width, UW = umbilicus width and W = width.

and *Cyclophorus friesianus* Moellendorff, 1883 (GenBank accession number No. HM753719, HM753951, HM753893, HM754125). We choose these as outgroup because *Ptychopoma* is a sister group of *Cyclotus* (Lee et al., 2008). However, the use of a closely related sister group as outgroup may not always lead to the correct phylogenetic tree (Lyons-Weiler et al., 1998; Sanderson & Shaffer, 2002), and it can be solved by using multiple outgroups. We use *Cyclophorus* that is outside of, but closely related to the ingroup as additional outgroup. Sequences were assembled and edited using Bioedit 5.0.9 (Hall, 1999). All alignments were performed using Clustal X (Thompson et al., 1997) and were manually proofread.

All data sets were subjected to maximum likelihood (ML) analyses using PhyML 3.0 (Guindon & Gascuel, 2003) and to neighbor-joining (NJ) analysis using MEGA 4.0 (Tamura et al., 2007). The substitution model used for the data set corresponded to the TVM+I+G model. It was the best models found using jModelTest 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008). Before model fitting, the full-length sequences were tested to confirm that there was no significant heterogeneity in base frequencies across taxa (in COI: $X^2 = 10.91$, df = 108, P = 1; in 16S rRNA: $X^2 = 37.17$, df = 132, P = 1). NJ bootstraps consisted of 1000 iterations. The reliability of the ML trees was estimated by the approximate likelihood ratio test (aLRT) (using a custom-defined model, base frequencies: A = 0.3488, C = 0.1561, G = 0.1150, T = 0.3802) using PhyML 3.0. Gaps (from insertions/deletions) were treated as missing data.

Neutrality was tested using Tajima's D (COI: D = -0.53034, P > 0.10; 16S rRNA: D = -0.75349, P > 0.10) and Fu & Li's F statistic (COI: F = 0.23658, P > 0.10; 16S rRNA: F = 0.26132, P > 0.10) (Fu & Li, 1993; Tajima, 1989). There were no selective effects on COI and the 16S rRNA gene.

Associations of the genetic, morphological and geographic distance between populations were explored through Mantel tests in XLSTAT (2010) on the pairwise genetic p-distance, pairwise similarity of shell characters (using Euclidean distance calculated by Primer 5.1.2 (2000)), and pairwise geographic distance. On the pairwise population fixation indices derived from AMOVA analysis (using Arlequin 3.5) (Excoffier & Lischer, 2010).

RESULTS

Morphological Analysis

The first three principal components accounted for 49.59%, 22.38% and 9.68% of the total variation in the matrix. The loadings of characters on the principal components reflected distinct groups of shell traits: umbilicus proportion (PC1), outer lip proportion (PC2), and aperture proportion (PC3). The *C. adamsi* group (sampling sites 1–8) was fully separated from the other four taxa (sampling sites 9–27) along the two axes, but *C. peraffinis, C. dilatus, C. taivanus* and *C. diminutus* partially overlapped (Fig. 3).

Phylogenetic Analysis

Alignment of the COI + 16S rRNA datasets yielded 1,375 characters, of which 544 were variable sites. Of these 544 positions, 481 (88.42%) were parsimony informative. The average p-distance among haplotypes was 0.078. Sequence divergence among the haplotypes ranged from 0.001 to 0.126.

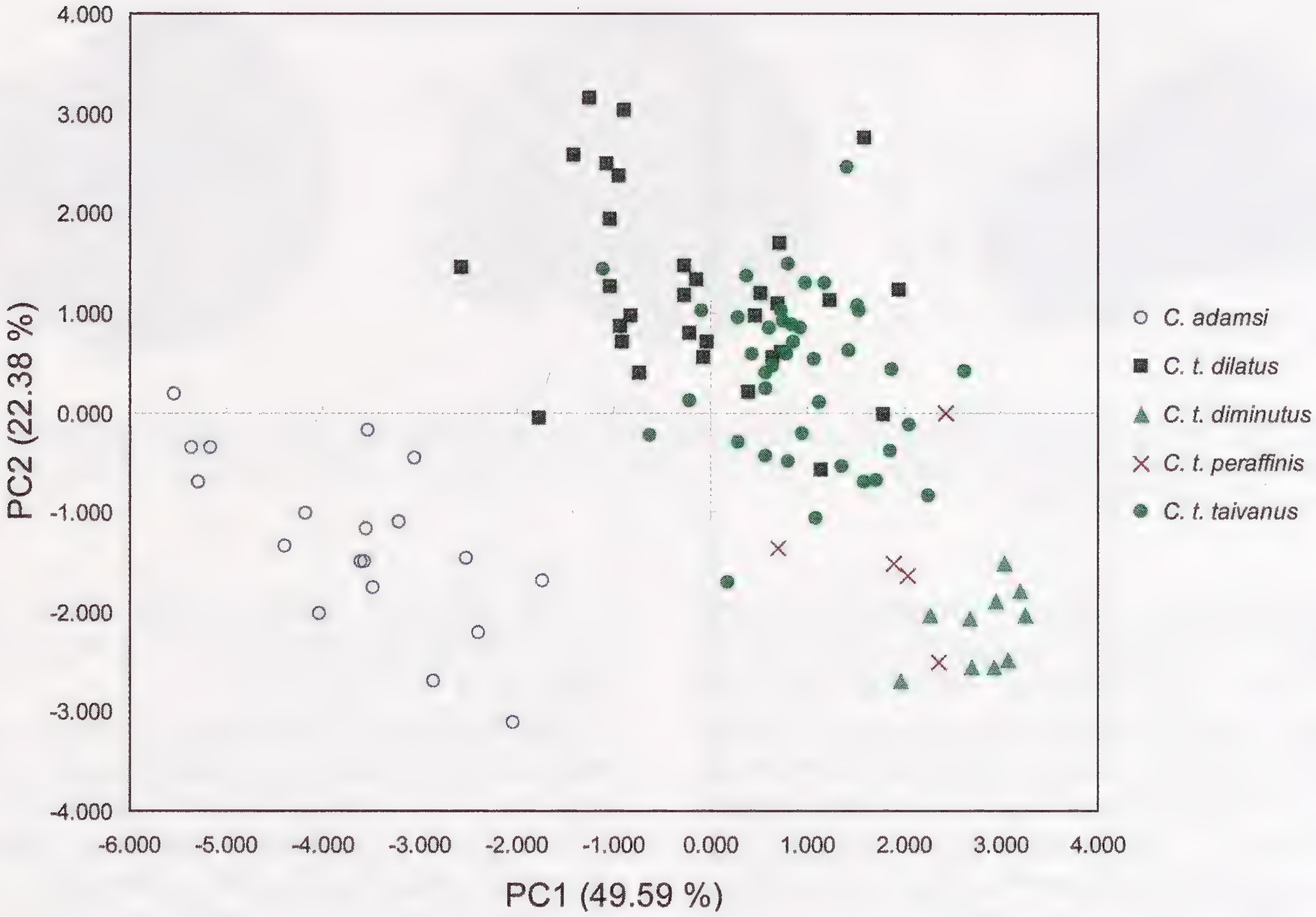

FIG. 3. Scatter plot of PCA scores on the first two axes of principal component analysis of *Cyclotus* shell morphological variables. Axis 1 accounts for 49.59% and axis 2 for 22.38% of the total variance. Different symbols illustrate the sampling sites of the five taxa, as described in Fig. 1.

The COI + 16S rRNA cladograms constructed using different tree-building methods exhibited a similar topology, differing only minimally on the terminal branches; therefore, we present only the ML tree here (Fig. 4). The topology of the COI + 16S rRNA gene tree strongly suggested the presence of two fundamental haplotype clades named A and B. Analyzing each clade separately, the average sequence divergence between haplotype clades A and B was 0.119 changes per site, which is relatively high compared with the changes within clades A and B (0.01 and 0.051, respectively). *C. adamsi* was clustered in clade A, while clade B comprised *C. peraffinis, C. dilatus, C. taivanus*, and *C. diminutus*.

The very high F_{ST}s among clade A and clade B, derived from AMOVA analysis indicated the absence of gene flow between these two clades. Beside, the high F_{ST} indicated limited gene flow between them.

Environmental Characterization of Sites

A PCA scatter plot of the sampling sites shows that the sampled populations primarily comprise (axis 1, 44.03% of total variation) an environmental gradient from north Taiwan, with a low temperature, to south Taiwan, with a high temperature (Fig. 5). The second most important (axis 2, 32.42%) is a gradient with a more changeable temperature (annual range of monthly normal temperature), lower warm-season precipitation, and higher cold-season precipitation from north to south Taiwan.

When the results of molecular species characterization are superposed on the PCA scatter plot (Fig. 5), it becomes clear that clade A populations are mainly situated in the climate region with the variable temperature, lower warm-season precipitation and higher cold-season precipitation.

Mantel Test

There are four subspecies with variable shell morphology in clade B. Therefore, we performed the Mantel test to investigate the correlation between molecular data, morphology and geographic distribution.

The Mantel test to examine the association between the geographic distance separating

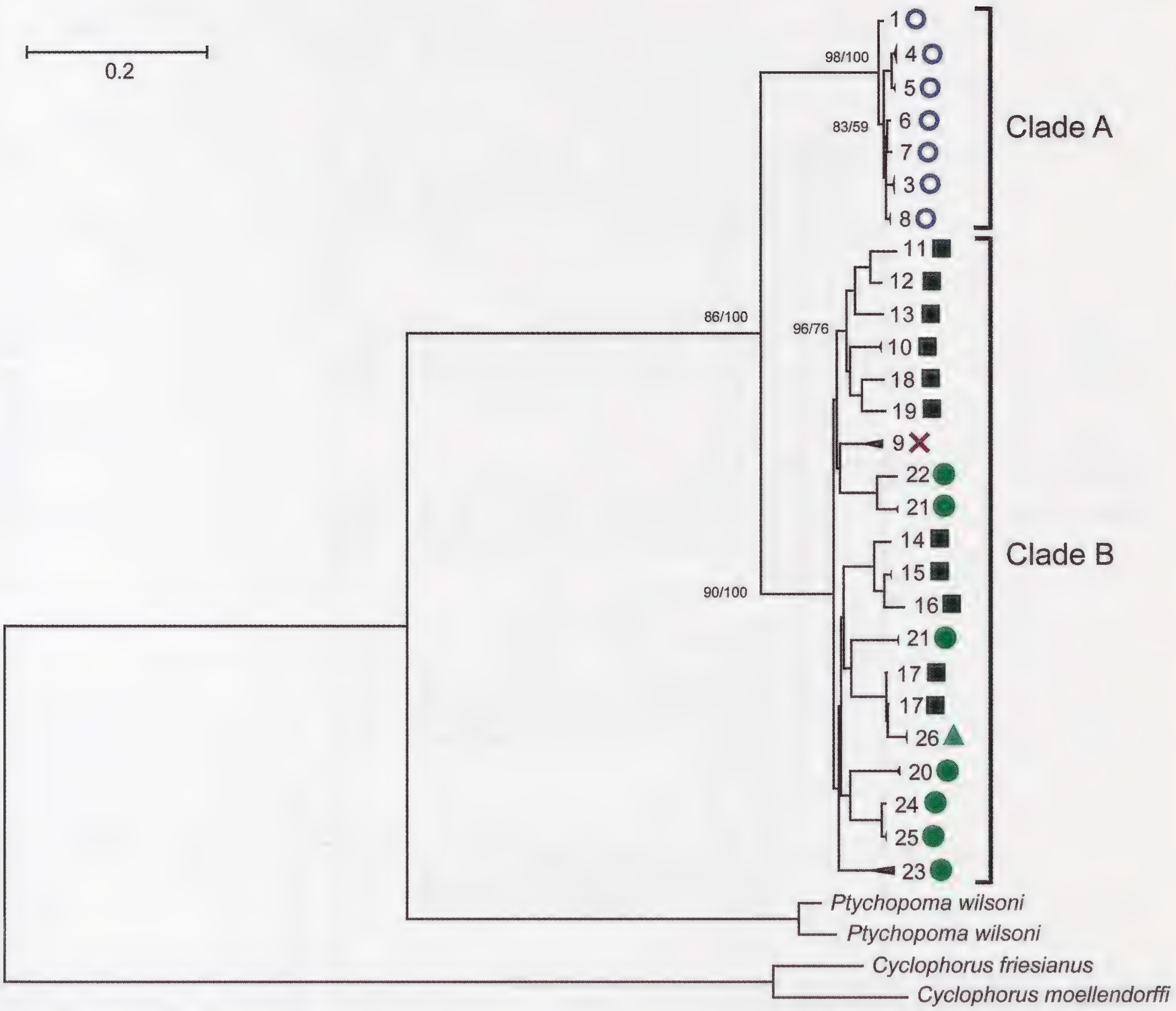

FIG. 4. Molecular phylogeny of *Cyclotus* based on concatenated COI and 16S rRNA gene sequences, with aLRT score / NJ bootstrapon node. Branch tips are labeled with the sampling site numbers and the five taxa. Open circle: *C. adamsi*; solid square: *C. t. dilatus*; solid circle: *C. t. taivanus*; solid triangle: *C. t. diminutus*; X: *C. t. peraffinis*. Some detail branchs are compressed in terminal triangles.

populations and the molecular pairwise p-distance was significant (COI + 16S p-distance / geographic distance Mantel test, $r = 0.253$, $P < 0.0001$). In contrast, there were no correlations between the molecular pairwise p-distance and overall morphological similarity (COI +16S p-distance / morphological similarity Mantel test, $r = 0.038$, $P = 0.436$). The Mantel test was also performed between the pairwise geographic distance and the overall morphological similarity matrix, and a significant correlation between them was observed ($r = 0.368$, $P < 0.0001$).

PLS Analysis

PLS analysis detected a significant correlation between shell traits and environmental variables of clade B for the respective sampling sites (Table 2). We found a negative correlation between lower cold-season precipitation and outer lip breadth, a positive correlation between lower cold-season precipitation and degree of whorl increase, and a positive correlation between lower cold-season precipitation and whorl number.

DISCUSSION

Two Evolutionary Lineages in *C. taivanus* ssp.

Morphologic and genetic variation within and among populations was detected in the present survey over the range of the taxon described as

TABLE 2. Correlations between shell traits and environmental variables in *C. taivanus* group (clade B in Fig. 4).

Shell trait	Correlation							
	Warm season mean temperature	Cold season mean temperature	Annual mean temperature	Annual range of monthly normal temperature	Warm season amount of precipitation	Cold season amount of precipitation	Annual amount of precipitation	Altitude
size (H × W)	-0.032	-0.337	-0.198	0.161	0.280	-0.527	-0.388	0.175
tallness/flatness (H/W)	0.026	-0.003	0.005	0.037	0.070*	-0.081*	-0.053*	0.033
aperture proportion (AW/W)	-0.255	-0.454	-0.399	0.118**	0.189**	-0.420*	-0.359**	0.175
aperture shape (AW/AH)	0.247	0.422	0.368	0.084	-0.316	0.463	0.252	-0.161*
umbilicus proportion (UW/W)	0.337	0.423	0.423	0.045	-0.218	0.398	0.275	-0.132
outer lip proportion (LW/AW)	-0.406	-0.599	-0.569	0.061**	0.265**	-0.598**	-0.512**	0.181
flatness degree of body whorl (BH/BW)	0.025	-0.130*	-0.059*	-0.102	0.215	-0.212*	-0.093*	0.191
degree of whorl increase ([W-UW-LW]/HN)	-0.039	-0.307	-0.185	0.085	0.334*	-0.487**	-0.227**	0.226
whorl number (HN)	-0.011**	-0.373**	-0.203*	0.234**	0.150**	-0.514**	-0.579**	0.107

*Indicates a significant correlation at the 5% level; **Indicates a significant correlation at the 1% level.
Warm season: May, June, July, August, September.
Cold season: November, December, January, February, March.

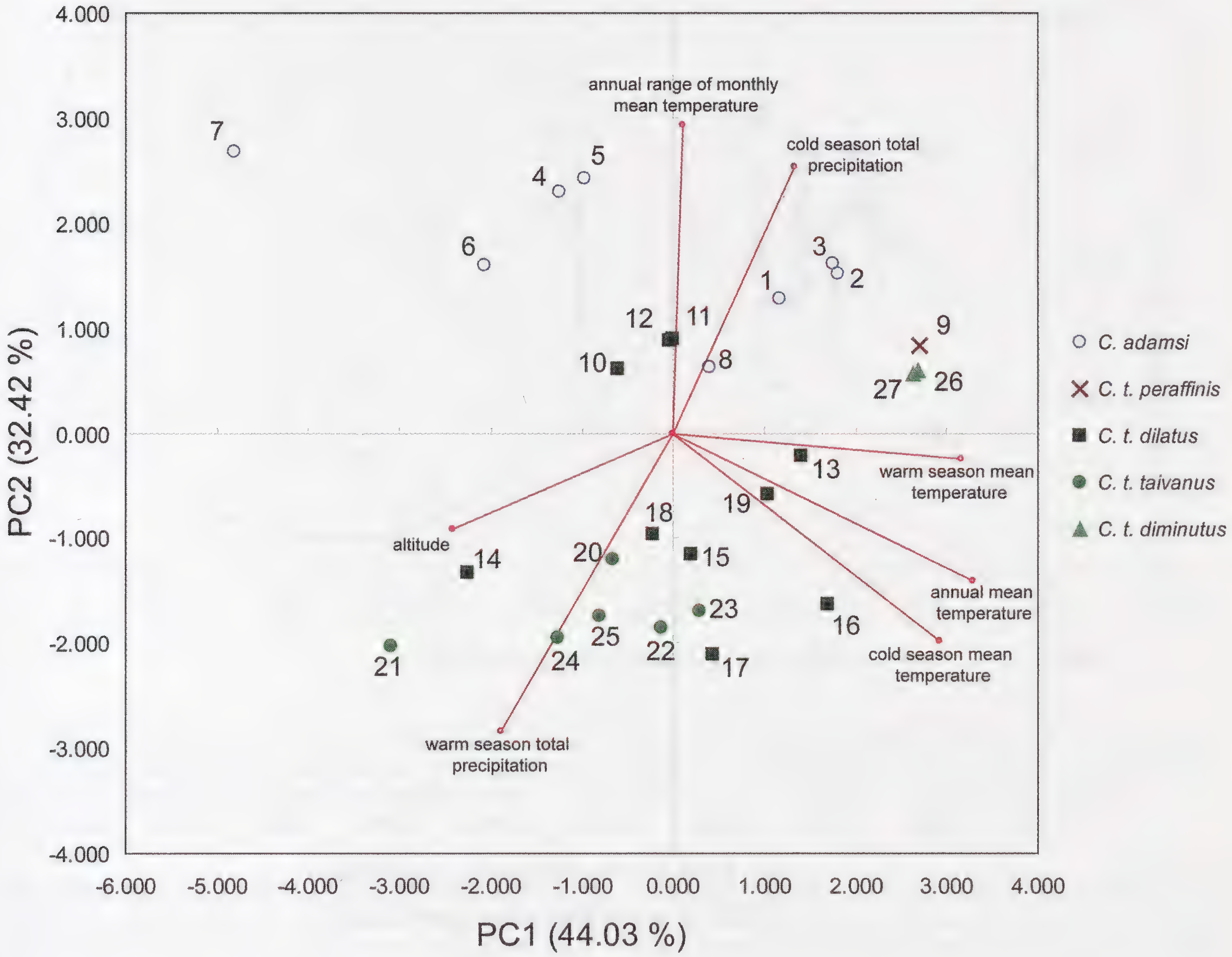

FIG. 5. Scatter plot of PCA scores on the first two axes of principal component analysis for environmental variables for 27 sites of *Cyclotus*. The correlation of variables with PCA axes is indicated by red line vectors. Sites where clade A populations were found are indicated in blue, clade B populations are indicated in brown, and series green color. Axis 1 accounts for 44.03% and axis 2 for 32.42% of the total variance. Different symbols illustrate the sampling sites of the five taxa, as shown in Fig. 1.

C. taivanus ssp. PCA revealed a clear distinction distribution of individuals in morph-space (Fig. 4), there were two highly divergent COI + 16S rRNA clades. The sequence divergence between the two clades was almost as high as that between other Cyclophoridae species (COI: 0.124 in *C. taivanus* ssp., 0.098–0.149 in other Cyclophoridae species; 16S rRNA: 0.107 in *C. taivanus* ssp., 0.083–0.087 in other Cyclophoridae species (Lee et al., 2008)). The average sequence divergence in COI and 16S rRNA among clade A and B equaled 0.124 and 0.107 changes per site, respectively. Owing to the lack of a calibration point, we have to assume that the two clades diverged approximately 5.4–5.5 million years ago, if the *Cyclotus* species has a similar mutation rate to that of Taiwan *Cyclophorus* (unpublished data; 2.28% and 1.95% per million years in COI and 16S rRNA, respecitively).

The presence of two highly-divergent genetic clades or evolutionary lineages is therefore in concordance with morphological differences. This led us to the conclusion that there are two separately evolving lineages in *C. taivanus* ssp. This view is strengthened by the observation that clade A, found only in sampling sites 1–8 from north Taiwan (Fig. 1). The absence of gene flow between clade A and clade B suggested reproductive isolation of the clades (Table 3). Most species concepts, reviewed in Wilkins (2009, 2011) would recognize clade A as a separate species in the light of the presented evidence. Among these contemporary species concepts, unified species concept of De Queiroz (2007) is the best concept to apply in our case. This species concept comprises the biological species concept without proving actual reproductive isolation, and equates species with separately

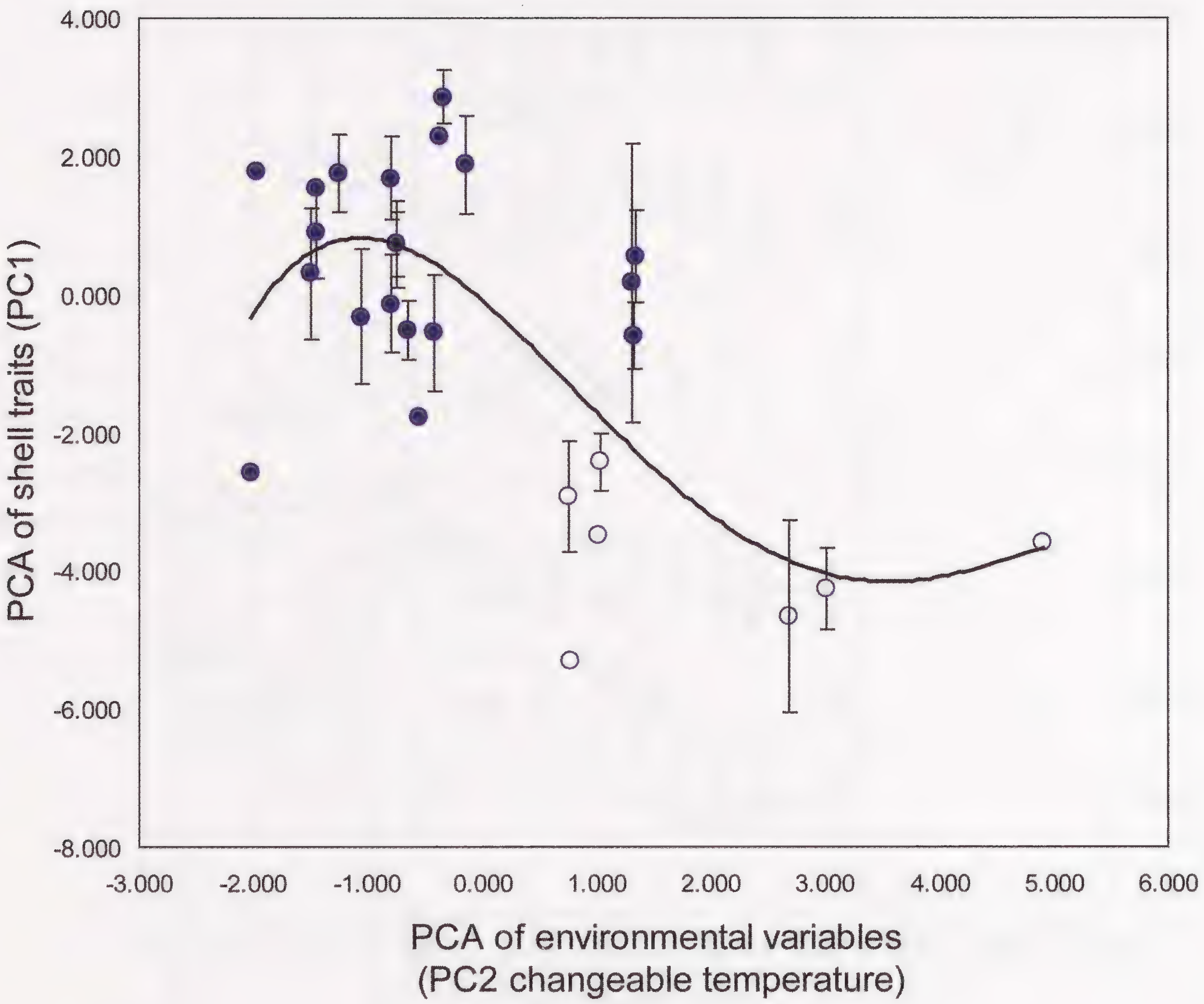

FIG. 6. Scatter plot of the environmental variables PC2 and shell traits PC1 averages (± SD). Open and solid circles indicate clades A and B, respectively. The trend line is the 4th degree multinomial.

TABLE 3. Population pairwise F_{ST}s derived from AMOVA analysis of *Cyclotus* using COI and 16S rRNA data, respectively.

	C. adamsi	*C. t. dilatus*	*C. t. peraffinis*	*C. t. taivanus*	*C. t. diminutus*
COI					
C. adamsi (clade A)	0.00000				
C. t. dilatus (clade B)	0.71136*	0.00000			
C. t. peraffinis (clade B)	0.87074*	0.22362	0.00000		
C. t. taivanus (clade B)	0.69307*	0.19160*	0.22257*	0.00000	
C. t. diminutus (clade B)	0.95352*	0.44126*	0.78815*	0.39114*	0.00000
16S rRNA					
C. adamsi (clade A)	0.00000				
C. t. dilatus (clade B)	0.78897*	0.00000			
C. t. peraffinis (clade B)	0.92744*	0.40061*	0.00000		
C. t. taivanus (clade B)	0.81128*	0.24100*	0.29710*	0.00000	
C. t. diminutus (clade B)	0.94225*	0.48908*	0.98652*	0.48698*	0.00000

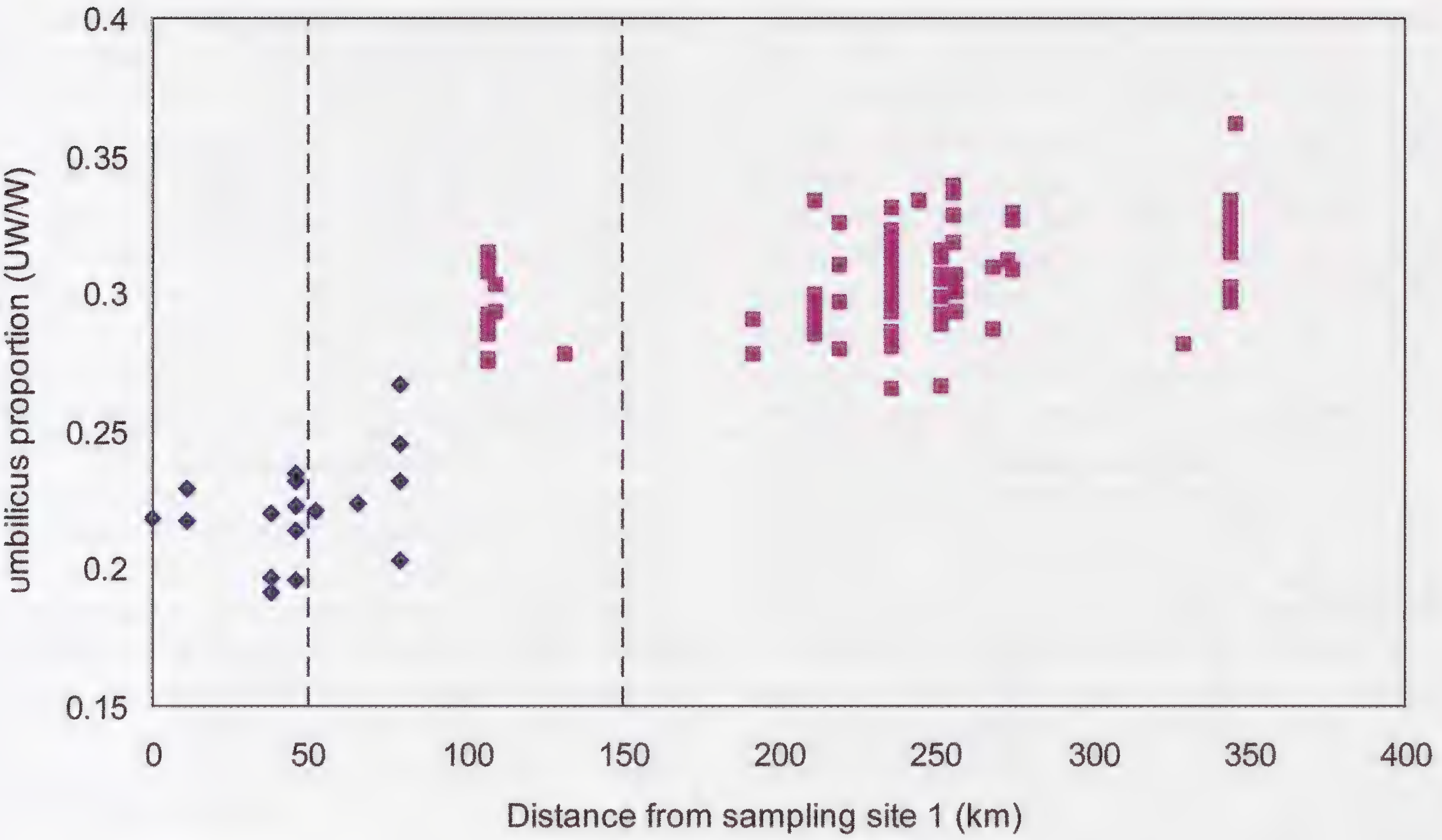

FIG. 7. The change in the umbilicus proportion of *C. adamsi* (blue rhombus) and the *C. taivanus* group (pink square) along the north to south population. Spots between dotted lines are measured from individuals around the contact zone.

evolving metapopulation lineages, which is appropriate in a case of allopatric relations. We recognized these two lineages (clade A and B) as different species according to the unified species concept, because the two lineages were reciprocally monophyletic and deeply divergent and showed a non-overlapping allopatric distribution, which indicates absence of gene-flow in these two lineages. For ease of description, and by comparing genetic clade and shell trait PCA data, we will refer to all individuals with the haplotype of clade A as *C. adamsi* (a valid species) and those with the haplotype of clade B as the *C. taivanus* group (*C. t. dilatus*, *C. t. diminutus*, *C. t. peraffinis*, and *C. t. taivanus*) hereafter.

Portions of the molecular and phenotypic variation were shown to be due to an obviously long separation of *C. adamsi* and the *C. taivanus* group. An initial survey of the geographical distribution of these two taxa indicated that they show no differences in habitat preferences. *Cyclotus adamsi* and the *C. taivanus* group are never sympatric. In the scatter plot of environmental variables PC2 and shell traits PC1, *C. adamsi* appears to have adapted well to an unstable, arid warm season, a moist cold season and a cold temperature, but the *C. taivanus* group has not (Fig. 6). Temperature may be a limiting factor in the distribution of *C. adamsi* and the *C. taivanus* group. Contrary to *C. adamsi*, the *C. taivanus* group with a flatter shell inhabits a warmer environment with a stable temperature. The warm and stable climate tends to have dense vegetation, and the flat shell is a supposed advantage such as less friction, when roaming over the ground in dense vegetation.

Hypothetical *C. taivanus* ssp. Speciation Model

It appears that a climatic gradient is responsible for the distribution patterns of the species (Fig. 5). Along this cline, *C. adamsi* occupies only sites with a moist winter and an arid summer climate. This type of climate appears to exclude the *C. taivanus* group. The actual level of water stress at a particular site depends strongly on the local microclimate, which may account for the observed intermingled pattern in the contact zone. Even though the sampling population was limited, there is probably a cline in terms of umbilicus proportion between populations near the contact zone (Fig. 7). Likewise, there are more or less clines in the other eight shell traits between populations near the contact zone (Fig. 8). Even though premating or postmating reproductive isolation may have evolved as a byproduct of ecological divergence, the ecological divergence appears

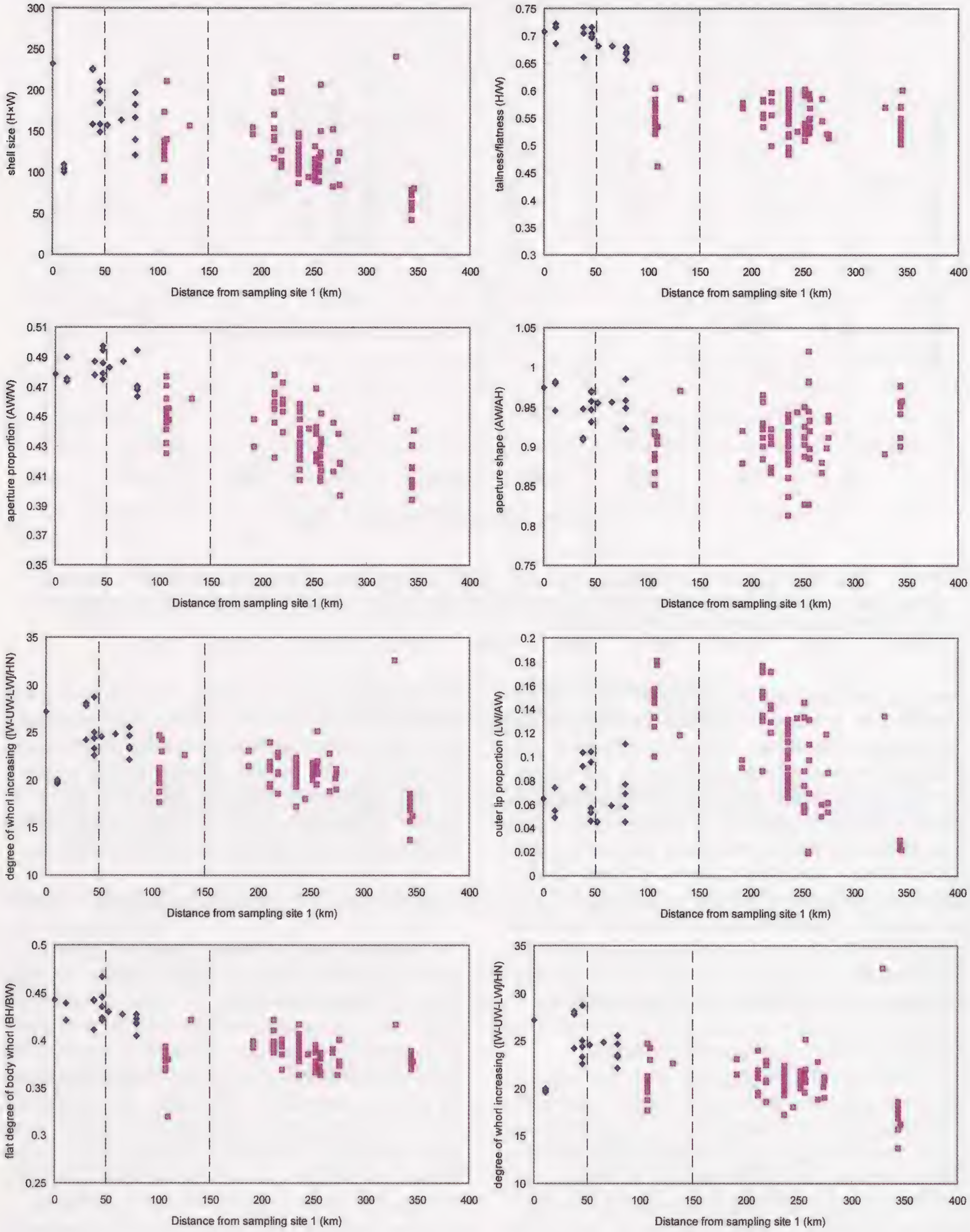

FIG. 8. The change in eight shell traits of *C. adamsi* (blue rhombus) and the *C. taivanus* group (pink square) along the north to south population. Spots between dotted lines are measured from individuals around the contact zone.

to be sufficient to prevent immediate contact, and therefore acts as an effective barrier to mating. The pattern of distribution along a climatic gradient suggests an ecotone divergence. Clines or ecotones are thought to promote the evolution of specialized adaptations in populations on either side of the border (Pfenninger et al., 2003). The speciation between *C. adamsi* and the *C. taivanus* group may be due to ecological speciation. An alternative hypothesis is that it is due to vicariance events and secondary contact. Parental individuals may never migrate between environments in the process of allopatric or parapatric speciation. However, ecological speciation may occur and the adaptations of local environment lead the evolution of reproductive isolation. The alternative model of speciation offers a much less likely scenario. There was no evidence to indicate an obstacle to dispersal around the contact zone in terms of historical geology.

Associations between Molecular and Morphological Variability within the *C. taivanus* Group

In the morphological PCA scatter plots of the *C. taivanus* group, all of its members partially overlap (Fig. 3). In particular, the shell morphology of *C. t. diminutus* and *C. t. peraffinis* are similar, and as expected their shell trait PCA scatter plots overlap (Fig. 3). However, there is limited gene flow between them (Table 3). The results of the Mantel tests indicated that the null hypothesis of no association between molecular p-distance and overall morphological similarity in shell traits could not be rejected within the *C. taivanus* group. This suggested that the observed shell trait differences have not evolved in concordance with the mitochondrial genome, and as a result the phenotypic population structure does not reflect the phylogenetic history of the populations.

Associations between the Environment and Morphological Variability within the *C. taivanus* Group

The results of PLS analysis showed that differences between populations in some traits covaried significantly with the long-term climatic conditions of the sampling site. We found that precipitation is correlated with some shell traits of the *C. taivanus* group. Cold-season precipitation and outer lip proportion are significantly negatively correlated; in contrast, warm-season precipitation and outer lip proportion are significantly positively correlated.

The same applied to whorl number, sampling sites with a greater annual precipitation which are associated with dry summer in Taiwan (Lin, 1990), being associated with a lower whorl number. This appeared astonishing at first, because a humid climate allows for greater feeding activity, and snails should therefore attain a greater number of whorls. A similar tendency towards smaller snails occurring in habitats with more rainfall was observed by Goodfriend (1986), and can possibly be explained as an adaptation of sexual maturity. There is no further whorl growth and forming an aperture lip when *Cyclotus* reaches sexual maturity. Reproducing earlier in a wetter cold season would offer the next generation the possibility of reaching a larger size before summer. Early reproduction could thus constitute a selective advantage, because a small size of juveniles of most snails results in high mortality in the dry summer of Taiwan (experience from field work). The ecological divergence probably appears rule of speciation in the case of *C. taivanus* ssp.. Nevertheless, the speciation process is not complete among *C. t. dilatus*, *C. t. diminutus*, *C. t. peraffinis*, and *C. t. taivanus*, and the continued adaptation to climatic pressure is a rule of the speciation process. However, further experiments are needed to determine whether the size/precipitation correlation in the *C. taivanus* group is due to phenotypic plasticity in response to the prevailing conditions or whether it has an adaptational significance.

In conclusion, we consider the phenotypic and phylogenetic divergence between the two identified lineages sufficiently large to propose the presence of two distinct evolutionary entities as valid species (*C. adamsi* and the *C. taivanus* group). The speciation process between *C. adamsi* and the *C. taivanus* group is complete, as evidenced by a lack of gene flow (Table 3). The reproductive isolation of the two taxa may be due to divergent selection in traits in different environments. In other words, the speciation model in this case is ecological speciation. However, more sampling sites around the contact zone should be contained in further studies. For the subordinate members of *C. taivanus*, there is no resolution in phylogeny analysis of the present investigation. To resolve the relationships in the *C. taivanus* group, more rapid evolved molecular marker is needed for further study. In addition, hypotheses regarding correlations between *C. taivanus* group divergence and environmental variables could be tested in future studies.

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